REMARKS

FORMAL MATTERS:

Claims 34, 40, 45-66 and 69-74 are pending in this application. Claims 71-74 have been previously withdrawn from consideration. As such, claims 34, 40, 45-66 and 69-70 are pending and under examination in this application.

Claim 59 has been amended to correct a typographical error. Support for this amendment can be found in Claim 13 as originally filed.

Claims 77-79 are added herein. Support for new claim 77 can be found, for example, in original claim 33. Support for new claim 78 can be found, for example, in original claim 39. Support for new claim 79 can be found, for example, in the specification at page 18, lines 3-6 and at page 55, line 14, to page 56, line 24.

As no new matter is added in these new claims, entry by the Examiner is respectfully requested.

INTERVIEW REQUEST

The Applicants herein request a personal interview with the Examiner to discuss the outstanding rejections in this case in view of the amendments and arguments presented herein. Applicants will contact the Examiner in due course to find a suitable date and time to conduct the interview.

REJECTIONS UNDER §101, UTILITY

Claims 34, 40, and 45-66, 69 and 70 have been rejected under 25 U.S.C. §101 as lacking patentable utility. The Applicants respectfully traverse this rejection.

The Office Action has reiterated that the claimed invention is allegedly not supported by either a specific and substantial asserted utility or a well-established utility. In particular, the Office Action states:

> Each method of screening requires an orphan GPCR that has been associated with a disease or disorder. However, each claimed method lacks specific and substantial utility because the orphan GPCRs to be used are associated with an *unspecified* disease or disorder. (see page 5 of the Office Action)

The pending claims are directed to methods for screening orphan receptors. The claimed methods will work with any orphan receptor that is constitutively active or can be made to be constitutively active. Thus, the claims are not, and should not be, limited to any particular orphan receptor. Because the claims are not limited to a particular orphan receptor, they are likewise not limited to a particular disease or disorder. The claims recite that the skilled person will have in hand an orphan receptor of interest associated to their satisfaction with a disease or disorder.

Generally, the claims of the subject application are directed to a "research tool" or enabling technology which plays an important role in developing a biopharmaceutical end product (compounds), where without it, the end product would either not have been found or found only after a great deal of effort and expense. As of the filing date of the invention, orphan receptors were not screened until they had been "de-orphanized" (i.e., an endogenous ligand had been identified). However, finding an endogenous ligand for an orphan receptor was, and still is, very expensive, time consuming and oftentimes unsuccessful. The claimed methods provide a novel and inventive way to screen for compounds that modulate an orphan receptor's activity without first de-orphanizing the receptor (i.e., identifying the endogenous ligand).

The ability to use the claimed methods on any orphan receptor demonstrates the real world usefulness of this invention. In fact, the Assignee of the subject application, Arena Pharmaceuticals, was able to raise venture capital for research based on this technology (called CART for constitutively activated receptor technology). Applicants have provided herein as Exhibit A, a press release dated February 22, 1999, which announces successful completion of a $17 million private offering. In the press release Arena's president and CEO is quoted as saying "we view the substantial over-subscription for our Series D Preferred Stock as a good indication of the support that our investors have in Arena and our CART™ Technology". The press release goes on to further describe the CART technology.

Applicants also provide another press release from November 15, 1999, announcing a collaboration between Arena Pharmaceuticals and Neurocrine Biosciences for use of Arena's constitutive activation technology to three orphan GPCRs (see Exhibit B). A quote form the vice president of drug discovery at Neurocrine Biosciences indicates that "Orphan receptors provide great opportunities for new discoveries in biology and neuroscience and can become novel drug targets. Neurocrine has discovered several orphan receptors and Arena's proprietary technology will allow us to maximize our evaluation of these potential drug targets and provide strategies to screen these receptors for small molecule agonists and antagonists."

Clearly, both investors and other biotechnology companies appreciated the "real world" utility of Arena's CART technology.

The Examiner summarizes his position by quoting *In Re Fisher* (76 USPQ2d 1225 (CA FC 2005)), which concludes by stating: "Simply put, to satisfy the 'substantial' utility requirement, an asserted use must show that that claimed invention has a significant and presently available benefit to the public". Based on the discussion above, the Applicants respectfully submit that, in contrast to the Examiner's assertion, they have shown that the claimed invention has a significant and presently available benefit to the public, and therefore have fully satisfied the requirements of 35 U.S.C. §101. As such, withdrawal of this rejection is respectfully requested.

REJECTIONS UNDER §112, ¶1 (ENABLEMENT)

Claims 34, 40 and 45-66, 69 and 70 are rejected as not meeting the "how to use" part of the enablement requirement of 35 U.S.C. § 112, first paragraph.

The basis for this rejection is the Examiner's contention that the claims are not supported by a patentable utility.

As such, it is believed that this rejection has been adequately addressed in the discussion in the preceding section of this response.

In view of the discussion in the preceding section of this response, Applicants respectfully request withdrawal of this rejection.

REJECTIONS UNDER §112, ¶1 (WRITTEN DESCRIPTION)

Claims 34, 40 and 45-66, 69 and 70 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement.

Applicants note with thanks that the Examiner has acknowledged the specification does provide an example of an orphan GPCR (GPR3) that is associated with a disease (epilepsy). However, the Examiner does not consider this example to provide sufficient description for the genus of orphan GPCRs that are associated with diseases or disorders. The Applicants respectfully disagree.

First, the Applicants note that in addition to the working example of GPR3, the specification provides teaching for associating receptors and diseases or disorders. For example, see page 33, line 20, to page 34, line 12 and page 75, line 20, to page 76, line 1, excerpts from which are provided below:

> Once it has been appreciated that: 1) inverse agonists to orphan receptors can be identified-by the methodologies of this invention; and 2) that such inverse agonists are ideal candidates as lead compounds in drug discovery programs for treating diseases related to the receptors, a search, impossible in the prior art, for treatments to diseases becomes enabled by this knowledge. For example, scanning both diseased and normal tissue samples for the presence of a receptor now becomes more than an academic exercise or one which might be pursued along the path of identifying an endogenous ligand... The presence of the receptor in a diseased tissue, or the presence of the receptor at elevated concentrations in diseased tissue compared to a normal tissue strongly can be preferably utilized to identify a correlation with that disease.
>
> The data support the position that the present invention effectively and efficiently allows for direct identification of inverse agonists against a receptor for which the endogenous ligand is unknown. Because of this technology, correlating the distribution of orphan receptors in specialized tissue and/or correlating the presence of such receptors with specified diseases allows for a rational approach to the development of a pharmaceutical composition(s) for such diseases.

Second, Applicants further submit that at the time of filing other orphan receptors were known in the literature that had been associated with diseases and disorders. Applicants submit herewith as Exhibit C three different references evidencing this fact: (i) Liao et al., *The Journal of Experimental Medicine* (June 2, 1997) vol. 185, p. 2015-2023, entitled "STRL33, A Novel Chemokine Receptor–like Protein, Functions as a Fusion Cofactor for Both Macrophage-tropic and T Cell Line–tropic HIV-1"; (ii) Alkhatib et al., *Nature* (July 17, 1997) vol. 388, p. 238, entitled "A new SIV co-receptor, STRL33"; and (iii) Farzan et al., *The Journal of Experimental Medicine* (August 4, 1997) vol. 186, p. 405-411, entitled " Two Orphan Seven-Transmembrane Segment Receptors Which Are Expressed in CD4-positive Cells Support Simian Immunodeficiency Virus Infection".

Liao et al. describe the identification of a novel human gene, STRL33, which encodes an orphan GPCR having sequence similarity to chemokine receptors and to chemokine receptor–like orphan receptors. STRL33 is expressed in lymphoid tissues and activated T cells, and is induced in activated peripheral blood lymphocytes. In this reference, Liao et al. demonstrate that, in contrast with the major known cofactors CXCR4 and CCR5, STRL33 can function with CD4 to mediate fusion with cells bearing HIV-1 *Env* proteins from both T cell-tropic and macrophage-tropic HIV-1 strains. Therefore, Liao et al. disclose a human orphan GPCR associated with the infectivity and pathology of the virus that causes AIDS.

Alkhatib et al. describe further studies with the orphan receptor STRL33, this time in studies with simian immunodeficiency virus (SIV). Specifically, Alkhatib et al. show that transfection of STRL33 into Jurkat cells renders them competent for infection with SIV, demonstrating that this orphan receptor is a co-receptor for SIV. This activity has relevance to human AIDS apart from the general parallels between the human and simian systems, as SIV is phylogenetically thought to be the immediate progenitor of HIV-2, a virus known to cause AIDS in humans. Additionally, this study provides clues to understanding how individuals who are homozygous for an inactivating deletion in the CCR5 gene, and therefore thought to be resistant to HIV infection, are nonetheless infected with HIV-1. Specifically, HIV may rely on alternative co-receptors, including orphan receptors like STR33.

Farzan et al. disclose that two orphan seven-transmembrane receptors, gprl and gpr15, serve as coreceptors for SIV, and are expressed in human alveolar macrophages. Farzan et al. go on to find that gpr15 (the more efficient SIV coreceptor of these orphan receptors) is also expressed in human CD4 + T lymphocytes and activated rhesus macaque peripheral blood mononuclear cells. These results underscore the potential diversity of seven-transmembrane receptors that are used as entry cofactors by primate immunodeficiency viruses, including orphan receptors gprl and gpr15.

In view of the description of a working example of an orphan GPCR associated with a disease in the subject specification, the teachings in the specification, and the state of art at the time of the invention (as represented by the three references described above), the Applicants submit that the written description requirement is fully satisfied for the claims of the subject application. Withdrawal of this rejection is thus respectfully requested.

CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order numberAREN-001CIP.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: November 13, 2007

By: /David C. Scherer, Reg. No. 56,993/
David C. Scherer, Ph.D.
Registration No. 56,993

Enclosures:

Exhibit A – press release dated February 22, 1999 (1 page)
Exhibit B – press release dated November 15, 1999 (2 pages)
Exhibit C – (i) Liao et al., *The Journal of Experimental Medicine* (June 2, 1997) vol. 185, p. 2015 (9 pages); (ii) Alkhatib et al., *Nature* (July 17, 1997) vol. 333, p. 238 (1 page); and (iii) Farzan et al., *The Journal of Experimental Medicine* (August 4, 1997) vol. 186, p. 405 (7 pages).

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Exhibit A

Arena Pharmaceuticals, Inc. Announces Successful Completion of $17 Million Private Offering

SAN DIEGO, Feb. 22 /PRNewswire/ -- Arena Pharmaceuticals, Inc. (Arena(TM)), a privately-held biotechnology company, has announced the successful completion of a private offering through the sale of its Series D Preferred Stock. Arena had intended to raise $10 Million through this offering; gross proceeds from the sale of its Series D Preferred Stock amounted to $17 Million. The placement agent for the offering was ING Baring Furman Selz LLC (New York, NY). Co-lead investors for the offering were MPM Asset Management, LLC (Cambridge, MA) and International Biomedicine Management Partners, Inc. (Basel, Switzerland). Other institutional investors were Tripos, Inc. (Nasdaq: TRPS), OrbiMed Advisors, LLC (New York, NY) and A.M. Pappas & Associates (Durham, NC).

"We are very pleased with not only the amount of money that was raised by the sale of Arena Series D Preferred Stock, but also the quality of the Series D investors," noted Jack Lief, Arena's President & CEO. "As is well known within the industry, the past year has been a particularly difficult period for start-up companies to raise money, such that we view the substantial over-subscription for our Series D Preferred Stock as a good indication of the support that our investors have in Arena and our CART(TM) Technology." Proceeds from the offering will be used to fund Arena's on-going research and development activities, provide working capital and for other general corporate purposes.

Founded in April 1997, Arena is primarily focused on the discovery and development of novel therapeutic modulators of G protein-coupled receptors (GPCR), using its proprietary CART Technology. CART allows for the direct identification of such modulators at these receptors in a ligand-independent manner, making the technology particularly useful with respect to the over 2,000 orphan GPCR targets that are estimated to be a part of the human genome.

Such ligand independent screening is made possible by genetic alteration of receptors, using proprietary, genetic cassettes. In addition to its proprietary platform technology, the Company has also in-licensed late-stage pre-clinical candidates for clinical studies. Arena's lead compound for Alzheimer's disease is currently in Phase I studies. The Company's 38,000 square-foot research and development headquarters are located at 6166 Nancy Ridge Drive, San Diego, CA 92121.

SOURCE:
Arena Pharmaceuticals, Inc.

CONTACT:
Jack Lief, President & CEO of Arena Pharmaceuticals, Inc., 619-453-7200

Exhibit B

Neurocrine Biosciences and Arena Pharmaceuticals Announce Receptor Collaboration

SAN DIEGO, Sept. 15 /PRNewswire/ -- Neurocrine Biosciences, Inc. (Nasdaq: NBIX) and Arena Pharmaceuticals, Inc. ("Arena"), a privately held biopharmaceutical company, today announced a collaboration involving the application of Arena's constitutive activation technology to three Neurocrine orphan G protein-coupled receptors (GPCRs). The collaboration also includes the opportunity for screening of the constitutively activated Neurocrine orphan GPCRs by Arena using Arena's in-house chemical library, or using Neurocrine's in-house chemical library, for the direct identification of small molecule modulators of the activated GPCRs. These modulators may be useful as leads for the development of GPCR-based therapeutics.

"Orphan receptors provide great opportunities for new discoveries in biology and neuroscience and can become novel drug targets. Neurocrine has discovered several orphan receptors and Arena's proprietary technology will allow us to maximize our evaluation of these potential drug targets and provide strategies to screen these receptors for small molecule agonists and antagonists," said Paul Conlon, Vice President of Drug Discovery at Neurocrine Biosciences.

Founded in April of 1997, Arena is primarily focused on the discovery and development of novel therapeutic modulators of GPCRs, using its proprietary CART Technology. CART allows for the direct identification of such modulators at these receptors in a ligand-independent manner, making the technology particularly useful with respect to the over 2,000 orphan GPCRs that are estimated to be a part of the human genome. Such ligand independent screening is made possible by genetic alteration of receptors, using routinely applicable, and proprietary, genetic cassettes.

Neurocrine Biosciences is a leading neuroscience company focused on the discovery and development of novel therapeutics for neuropsychiatric, neuroinflammatory and neurodegenerative diseases and disorders. The Company's neuroscience, endocrine and immunology disciplines provide a unique biological understanding of the molecular interaction between central nervous, immune and endocrine systems for the development of therapeutic interventions for anxiety, depression, Alzheimer's disease, insomnia, stroke, malignant brain tumors, multiple sclerosis, obesity and diabetes.

Neurocrine Biosciences, Inc. news releases are available free of charge though PR Newswire's Company News On-Call fax service. For a menu of Neurocrine's previous releases, or to receive a specific release via fax call: (800) 758-5804, ext. 604138, or use the Internet via http://www.prnewswire.com/.

In addition to historical facts, this press release contains forward looking statements that involve a number of risks and uncertainties. Among the factors that could cause actual results to differ materially from those indicated in the forward looking statements are risks and uncertainties associated with Neurocrine's research and development programs and business and finances including, but not limited to, risks and uncertainties associated with, or arising out of, drug discovery, pre-clinical and clinical development of products including risk that research may not generate development candidates, development candidates will not successfully proceed through early clinical trials or that in later stage clinical trials will not show that they are effective in treating humans; determinations by regulatory and governmental authorities; changes in relationships with strategic partners and dependence upon strategic partners for performance of clinical and commercialization activities under collaborative agreements including potential for any collaboration agreement to be terminated without any product success; uncertainties relating to patent protection and intellectual property rights of third parties; impact of competitive products and technological changes; availability of capital and cost of capital; and other material risks. A more complete description of these risks can be found in the Company's Form 10K for the year ended December 31, 1998 and the current form 10Q each of which should be read before making any investment in Neurocrine common stock. Neurocrine undertakes no obligation to update the statements contained in this press release after the date hereof.

SOURCE:

Neurocrine Biosciences, Inc.; Arena Pharmaceuticals, Inc.
Web Site: http://www.neurocrine.com/

Company News On Call: http://www.prnewswire.com/comp/604138.html or fax, 800-758-5804, ext. 604138

CONTACT:
Elizabeth Foster or Paul Hawran, 858-658-7600, both of Neurocrine Biosciences, Inc.;
or Jack Lief, President & CEO of Arena Pharmaceuticals, Inc., 858-453-7200

STRL33, A Novel Chemokine Receptor–like Protein, Functions as a Fusion Cofactor for Both Macrophage-tropic and T Cell Line–tropic HIV-1

By Fang Liao,* Ghalib Alkhatib,‡ Keith W.C. Peden,§ Geetika Sharma,* Edward A. Berger,‡ and Joshua M. Farber*

*From the *Laboratory of Clinical Investigation; ‡Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; and the §Laboratory of Retrovirus Research, Center for Biologics, Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892*

Summary

The chemokine receptors CXCR4, CCR2B, CCR3, and CCR5 have recently been shown to serve along with CD4 as coreceptors for HIV-1. The tropisms of HIV-1 strains for subgroups of $CD4^+$ cells can be explained, at least partly, by the selective use of G protein–coupled receptors (GPCRs). We have identified a novel human gene, *STRL33*, located on chromosome 3 that encodes a GPCR with sequence similarity to chemokine receptors and to chemokine receptor–like orphan receptors. *STRL33* is expressed in lymphoid tissues and activated T cells, and is induced in activated peripheral blood lymphocytes. When transfected into nonhuman NIH 3T3 cells expressing human CD4, the *STRL33* cDNA rendered these cells competent to fuse with cells expressing HIV-1 envelope glycoproteins (Envs). Of greatest interest, STRL33, in contrast with CXCR4 or CCR5, was able to function as a cofactor for fusion mediated by Envs from both T cell line–tropic and macrophage-tropic HIV-1 strains. *STRL33*-transfected Jurkat cell lines also supported enhanced productive infection with HIV-1 compared with control Jurkat cells. Despite the sequence similarities between STRL33 and chemokine receptors, *STRL33*-transfected cell lines did not respond to any in a panel of chemokines. Based on the pattern of tissue expression of the *STRL33* mRNA, and given the ability of STRL33 to function with Envs of differing tropisms, STRL33 may play a role in the establishment and/or progression of HIV-1 infection.

The seven transmembrane domain G protein–coupled receptors (GPCRs)[1] constitute a large family of proteins that signal in response to agonists as diverse as polypeptide hormones, neurotransmitters, odorants, light, and cytokines (1, 2). Within this superfamily is the group of receptors for the chemokines, cytokines that are produced by many tissues and cell types and whose best-described activities are as chemotactic factors for leukocytes, including lymphocytes (3). To date, 10 human receptors that signal in response to chemokines have been described, designated by the most recent nomenclature CXCR1–4 for the receptors for chemokines in the CXC subfamily and CCR1, 2A, 2B, and 3–5 for the receptors for chemokines in the CC subfamily. In addition, a number of orphan GPCRs have been identified whose sequences suggest that they may be receptors for as yet unidentified chemokines (4).

[1]*Abbreviations used in this paper:* β-Gal, β-galactosidase; Envs, envelope glycoproteins; FBS, fetal bovine serum; GPCRs, G protein–coupled receptors; HEK, human embryonic kidney; M-tropic, macrophage tropic; ORF, open reading frame; TCL-tropic, T cell line tropic; TIL, tumor-infiltrating lymphocytes; TMD, transmembrane domain.

Recent work has revealed novel relationships between chemokines–chemokine receptors and infection with HIV-1. Understanding these relationships has shed light on mechanisms of HIV-1 entry into cells and on the tropisms of HIV-1 strains for specific populations of $CD4^+$ cells. All HIV-1 strains can infect primary T cells. However, as a result of differences among their envelope glycoproteins (Envs), some HIV-1 strains are able to infect macrophages (M-tropic) but not immortalized T cell lines; some, among them laboratory-adapted strains, are able to infect immortalized T cell lines (TCL-tropic) much more efficiently than they infect macrophages; and some strains are readily able to infect both types of cells. Analysis of clinical HIV-1 isolates suggests that M-tropic strains are critical for establishing and maintaining infection (reviewed in references 5, 6).

The CC chemokines MIP-1α, MIP-1β, and RANTES were found to suppress infection of lymphocytes by M-tropic HIV-1 strains (7). This report was followed by the functional cDNA cloning of a cofactor (designated fusin) that, along with CD4, mediates fusion and entry of TCL-tropic HIV-1 strains (8). Fusin, previously identified as an orphan

GPCR, has recently been shown to be a receptor for the CXC chemokine SDF-1 and has been renamed CXCR4 (9, 10).

The discovery of the activity of CXCR4 was followed by the demonstration that chemokine receptor CCR5 can function as a cofactor with CD4 for cell fusion and/or viral entry and infection by M-tropic strains of HIV-1 (11–16). CCR5 has been demonstrated to play a critical role in the establishment of HIV-1 infection, because homozygosity for an allele encoding a fusion-defective CCR5, present in ~1% of the Caucasian population, is strongly associated with resistance to HIV-1 infection (17–20). Nonetheless, HIV-1 infection has been reported in an individual homozygous for the defective CCR5 gene (21), indicating that under some circumstances CCR5 is not essential for infection. Identifying the range of coreceptors that can be used by HIV-1 is important, particularly if therapies targeting specific coreceptors place the virus under selective pressure.

As part of studies on the roles of chemokines in lymphocyte biology, we designed experiments to identify novel chemokine receptors in human T cells. In this report, we describe the molecular cloning of a cDNA for *STRL33*, a gene encoding a GPCR related to known chemokine receptors that is expressed in lymphoid tissues and activated T cells, and is induced in activated PBL. We demonstrate that STRL33, in marked contrast with CXCR4 and CCR5, can function with CD4 as a cofactor for cell fusion mediated by Envs of both M-tropic and TCL-tropic strains of HIV-1 and we show that transfection with *STRL33* significantly enhances the ability of Jurkat cells to support productive infection with HIV-1. These data suggest that STRL33 may play a role in the establishment and/or the course of HIV-1 infection.

Materials and Methods

Cell Culture. Jurkat, SUP-T1, U937, human embryonic kidney (HEK) 293, HeLa, and NIH 3T3 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). CEM clone 12D7 was obtained from Dr. G. Poli (National Institute of Allergy and Infectious Diseases, National Institutes of Health). Tumor-infiltrating lymphocytes (TIL) R4, R8, F9, and B10, prepared from human melanomas, were obtained from Dr. J.R. Yannelli (National Cancer Institute). EBV414 is an EBV-transformed B lymphoblastoid cell line obtained from Dr. R. Siliciano (Johns Hopkins University). Jurkat, SUP-T1, CEM, U937, and EBV414 cells were grown in RPMI-1640 with 10% fetal bovine serum (FBS). Elutriated PBL and monocytes were obtained from a normal donor by the Department of Transfusion Medicine (National Institutes of Health), and PBL were purified further by banding on Ficoll–Paque. For activation, 5×10^6 PBL/ml were cultured with 10 μg/ml PHA-P and 1 μM ionomycin for 3 d before harvesting for RNA. 293 cells were grown in MEM plus 10% horse serum. HeLa and NIH 3T3 cells were grown in DMEM with 10% FBS. TIL were grown in either RPMI-1640 with 10% FBS or in AIM-V (GIBCO BRL, Gaithersburg, MD), in each case supplemented with 500 U/ml IL-2, and the cells were stimulated periodically with 250 ng/ml PHA plus irradiated allogeneic PBMC.

Cloning of STRL33 cDNAs. Total RNA was prepared from the F9 TIL using TRIzol reagent (GIBCO BRL, Gaithersburg, MD), poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Collaborative Biomedical Products, Bedford, MA), and first-strand cDNA was synthesized using oligo(dT) primers and the SuperScript Preamplification System (GIBCO BRL) according to the instructions of the manufacturer. For amplification, primer pools were designed based on transmembrane domain (TMD) II and TMD VII amino acid sequences from the human sequences for IL-8RA, IL-8RB, CCR1, and CCR2 and the murine homologues of IL-8RB and CCR1 and were 5′-GA(T/C)(C/T)TI(C/T/G)TITT(T/C)(G/T/C)(C/T)I(T/C/A)TIACI(T/C)TICC, and 5′-CCIA(T/C)(A/G)AAI(G/A)(C/T)(A/G)TAIA(T/A/G)IA(G/A/T/C)IGG(A/G)TT, respectively. Amplifications were done with cDNA synthesized from 0.015 μg of poly(A)$^+$ RNA, with 1.5 μM of each primer pool in a 20 μl reaction volume with Taq polymerase and reagents from Perkin Elmer (Norwalk, CT), according to the instructions of the manufacturer. PCR was done using 30 cycles of denaturation at 94°C for 0.5 min, annealing at 45°C for 2 min, and chain extension at 72°C for 1.5 min.

1 μl from the first PCR was used in a second PCR done identically to the first and the products of the second reaction were separated on a 1.5% agarose gel from which fragments of the predicted size of ~670 bp were purified and inserted by blunt end ligation into the vector pNOTA/T7 (5′ 3′ Prime, Inc., Boulder, CO). 88 ampicillin-resistant bacterial transformants were picked and, to eliminate known sequences, hybridizations were done with radiolabeled oligonucleotide probes for receptors *CCR1*, *CCR2*, *CCR3*, *CXCR4*, *BLR1*, *EBI1* and *STRL22*. Among the inserts in the nonhybridizing colonies was a novel sequence designated *STRL33*.

Using poly(A)$^+$ RNA from F9 TIL a λ ZAP Express (Stratagene, La Jolla, CA) cDNA library was prepared according to the instructions of the manufacturer. 1.4×10^6 recombinant phages from the nonamplified library were screened using a radiolabeled *STRL33* probe. 10 positive phages were plaque-purified and the pBK-CMV (Stratagene, La Jolla, CA) plasmids containing *STRL33* inserts were recovered by in vivo excision according to the instructions of the manufacturer. Manual and/or automated dideoxy sequencing was done for the entire cDNA clone *STRL33*.1, the 5′ nontranslated region of clone *STRL33*.2, the 5′ nontranslated region and open reading frame (ORF) of clone *STRL33*.3, and portions of other cDNA clones, some of which were obtained using RT–PCR.

Northern Blot Analysis. Total RNA was prepared as above. DNAs used for probes were the following: *IL8RA*, *IL8RB*, *CCR3*, *EBI1*, and *BLR1* genomic fragments and *CCR2B* cDNA obtained from Dr. P. Murphy (National Institute of Allergy and Infectious Diseases); *STRL33*, *CCR1*, *CCR4*, *CCR5*, *CXCR4*, and *CMKBRL1* cDNAs that we isolated either from our λ library or by RT–PCR from TIL mRNA; and an *STRL22* genomic fragment isolated as described (22). Hybridizations to leukocyte RNA were performed as described with washes in 0.1× SSC, 0.1% SDS at 50°C (23). Hybridizations with an oligonucleotide probe to 18S rRNA were as described (24). The blot of poly(A)$^+$ RNA from human tissues was obtained from Clontech (Palo Alto, CA). Hybridizations were done according to the instructions of the manufacturer with washes as described above. Autoradiography/fluorography was done using an intensifying screen.

Production and Analysis of STRL33-transfected Cell Lines. An EcoRI–EarI fragment containing the complete *STRL33* ORF was isolated from the pBK-CMV/*STRL33*.1 plasmid and inserted into pCEP4 (Invitrogen, Carlsbad, CA) and pCIneo (Promega Corp.,

Madison, WI). The pCIneo/*STRL33* DNA was transfected into HEK 293 cells by calcium phosphate precipitation (25) and the pCEP4/*STRL33* DNA and pCEP4 without a cDNA insert were transfected into Jurkat cells by electroporation (26). Selection was in 200 μg/ml hygromycin B (Sigma, St. Louis, MO) and 1 mg/ml G418 (GIBCO BRL, Gaithersburg, MD) for pCEP4-transfected cells and pCIneo-transfected cells, respectively. Individual colonies of resistant 293 cells were cloned and expanded and Jurkat lines were derived by limiting dilution after the electroporation. Lines expressing the highest levels of *STRL33* mRNA were used to test responses to chemokines using the fluorometric calcium flux assay as described (27), or for infection with HIV-1 as described below. Recombinant HuMig was obtained by infecting High Five cells of *Trichoplusia ni* (Invitrogen, Carlsbad, CA), as will be described elsewhere, and was purified by column chromatography as described (27). IP-10, MCP-1, MCP-2, MCP-3, RANTES, MIP-1α, MIP-1β, platelet factor 4, IL-8, and lymphotactin were purchased from Pepro Tech (Rocky Hill, NJ). MCP-4 was a gift from Dr. A. Luster (Harvard University). I309 and SDF-1 were gifts from R&D Systems (Minneapolis, MN).

Assays for Activity of STRL33 as a Fusion Cofactor. Assays were done using a vaccinia-based *Escherichia coli lacZ* reporter gene assay for fusion between two cell populations, one expressing an HIV-1 Env and the other expressing CD4 (28). Vaccinia-mediated expression of GPCRs was achieved by transfection of 7×10^6 NIH 3T3 cells using DOTAP lipofectin (Boehringer Mannheim, Indianapolis, IN) with 10 μg of plasmids containing DNA encoding the GPCRs linked to the bacteriophage T7 promoter. The plasmid vectors were pCIneo (Promega Corp., Madison, WI) for STRL33 and pCDNA3 for CXCR4 (8) and CCR5 (11); as a negative control, pCIneo lacking an insert was used. After 4–5 h, the transfected cells were infected at 10 PFU/cell with recombinant vaccinia viruses vCB-3 encoding human CD4 (29) and vTF7-3 encoding T7 RNA polymerase (30). A separate population of HeLa cells was coinfected with vaccinia virus vCB-21R–LacZ containing *lacZ* encoding β-galactosidase (β-Gal), under control of a T7 promoter (31) and one of the following Env-encoding vaccinia viruses: vCB-41 encoding the LAV (NL4.3) Env (32), vCB-39 encoding the ADA Env (32), vCB-28 encoding the JR-FL Env (32), vCB-32 encoding the SF-162 Env (32), vCB-43 encoding the Ba-L Env (32), vSC60 encoding the IIIB Env (Chakrabarti, S., and B. Moss, personal communication), and vCB-16 encoding the nonfusogenic Unc Env (32). For experiments using the 89.6 Env, before infection with vCB-21R–LacZ, HeLa cells were transfected with 10 μg of the pSC59 plasmid (Chakrabarti, S., and B. Moss, personal communication) containing DNA encoding the 89.6 Env (33), a gift of R. Collman (University of Pennsylvania School of Medicine). Infected cells were incubated overnight at 31°C. Duplicate samples of 10^5 NIH 3T3 target cells and 10^5 Env-expressing cells were mixed; after 2.5 h cells were lysed and β-Gal activity was measured as described (28).

Infectivity Assay for HIV-1. Virus stocks of HIV-1_{ELI} were prepared by transfection of 293 cells with molecular clone pELI1 (34), and the amount of virus was determined by RT activity (35). 2×10^6 cells of the Jurkat cell lines were infected with the amount of virus corresponding to 10^5 cpm of RT activity as described (35). Virus production was measured by the appearance of RT activity in the medium.

Results and Discussion

To identify novel chemokine receptors expressed in T cells, we used RT-PCR with poly(A)$^+$ RNA prepared from TIL line F9 and pools of degenerate primers based on conserved sequences in the TMDs of known chemokine receptors. We isolated a sequence encoding a novel GPCR, designated STRL33, for seven TMD receptors from lymphocytes clone 33. Southern blot analysis of human genomic DNA digested with BamHI, HindIII, and PstI revealed a single *STRL33* gene, and using *STRL33*-specific primers and DNA prepared from a panel of human–hamster hybrid cell lines, *STRL33* was localized to chromosome 3 (data not shown). This raises the possibility that *STRL33* is in the cluster of genes for chemokine receptors CCR1, 2, 3, and 5 at 3p21, although the CCR proteins show >50% amino acid identity among themselves, demonstrating significantly closer relationships than what is seen in comparisons with STRL33 (see below).

Screening of a nonamplified λ cDNA library prepared from F9 TIL revealed an abundance for *STRL33* mRNA of ~0.01%. 10 cDNA clones were isolated and by restriction enzyme digestion, three size classes were identified. Representative cDNAs from each class, *STRL33*.1, *STRL33*.2, and *STRL33*.3, respectively, were evaluated in more detail by restriction analysis and partial sequencing, revealing that size differences among the clones were due to 5′ nontranslated regions that differed not only in length but in their sequences. The complete sequence of *STRL33*.1, the sequence of the 5′ nontranslated region of *STRL33*.2, and the sequence of the 5′ nontranslated region and ORF of *STRL33*.3 have been submitted to GenBank with accession numbers U73529, U73530, and U73531, respectively. *STRL33*.1 contained 1,897 nucleotides, excluding the poly(A) tail, with an ORF encoding a predicted protein of 342 amino acids (Fig. 1). The predicted initiator codon was in a favorable context for initiation (36) and could be assigned unambiguously because, with the reading frame fixed by comparison with other GPCRs, it was the first ATG after an in-frame stop codon in cDNA *STRL33*.3, and it was also the first in-frame ATG in cDNAs *STRL33*.1 and *STRL33*.2. As noted above, ORF sequence was also determined from cDNAs other than *STRL33*.1, and the sequence of the entire *STRL33*.1 ORF was confirmed in independent clones. However, differences were noted between the ORFs of *STRL33*.1 and *STRL33*.3 at two positions: a second position change in the 25th codon of *STRL33*.3 (GAC → GCC), replacing D25 with A, and a silent third position change in the 103rd codon of *STRL33*.3 . The silent change in the 103rd codon of *STRL33*.3 was present in a second independent clone, whereas the 25th codon change has not been independently verified. We presume that at least the silent change represents a true polymorphism in the F9 TIL. The 5′ nontranslated regions of *STRL33*.1, *STRL33*.2, and *STRL33*.3 were 30, 135, and 1,462 nucleotides, respectively, and sequence comparisons revealed that differences among these regions were due, at least in part, to alternative splicing (data not shown). Not surprisingly, the long additional 5′ nontranslated sequence of *STRL33*.3 contained many ATG sequences (but no significant ORFs), suggesting that the *STRL33*.3 mRNA may not be translated efficiently, and although we could detect a

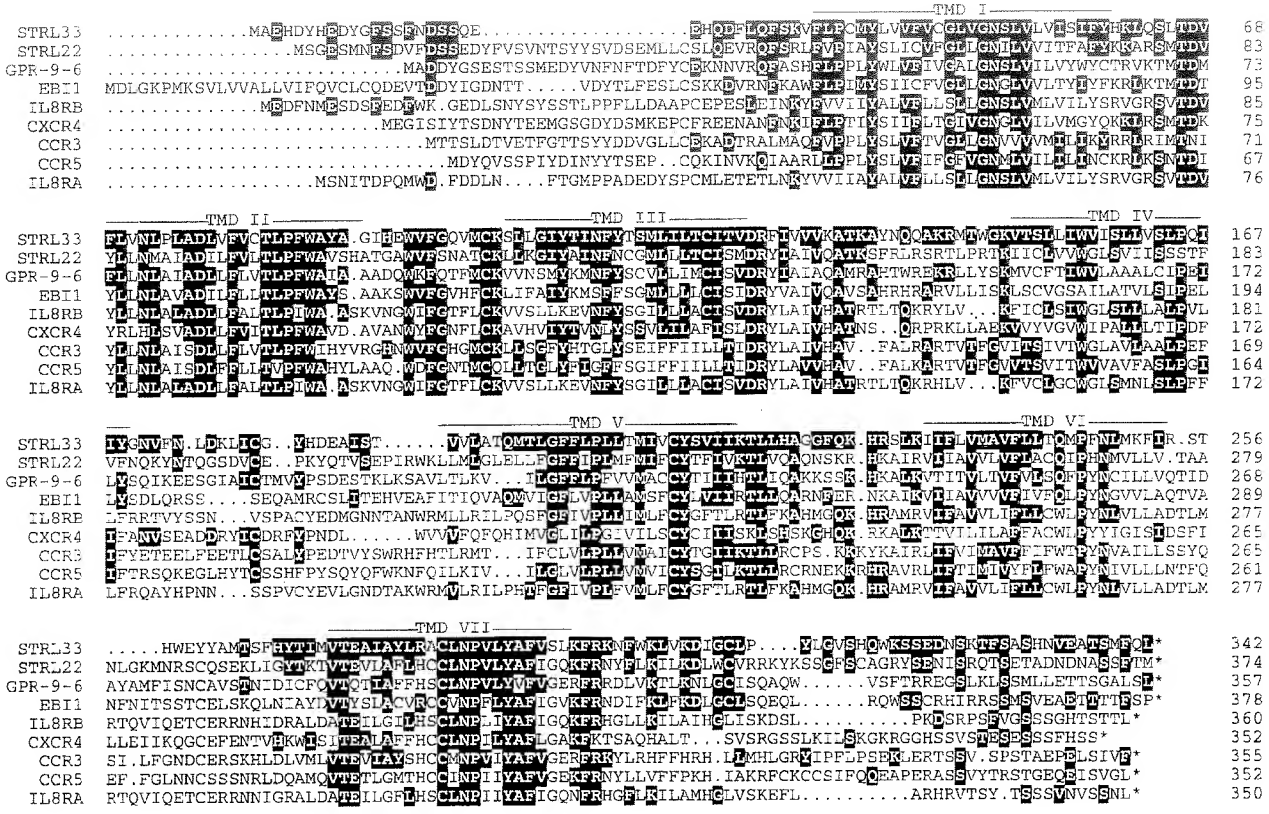


Figure 1. Alignment of the STRL33 predicted amino acid sequence with the selected GPCRs STRL22, GPR-9-6, EBI1, IL-8RB, CXCR4, CCR3, CCR5, and IL-8RA. Numbers at the right indicate the positions of the residues at the end of each line of sequence. Solid backgrounds highlight matches between STRL33 and the other receptors. Dots indicate gaps introduced for optimal alignments. Putative TMDs I–VII are indicated by bars. The alignments were generated using the PileUp program of the Wisconsin Sequence Analysis Package of Genetics Computer Group (Madison, WI).

STRL33.3-specific mRNA by Northern blot analysis (see below), the *STRL33*.3 cDNA may be derived from an incompletely processed mRNA. Extensive processing, yielding mRNAs with alternative 5′ exons, is well documented among the chemoattractant receptors (2), and incomplete processing of mRNAs in lymphocytes has been suggested as a possible mechanism for the regulation of translation (37).

Comparison of STRL33 with sequences in the GenBank database as of March 30, 1997, using BLAST (38) revealed no identical sequences but greatest similarity to orphan GPCRs and related chemokine receptors. Matches were found between sequences in the 3′ nontranslated region of *STRL33* and several sequences in the EST database for which no significant homologies had been identified. Alignments between STRL33 and selected related sequences is shown in Fig. 1. Percent identities between STRL33 and orphan receptors STRL22 (22), GPR-9-6 (GenBank accession number U45982), and EBI1 (39) are 37, 32, and 32%, respectively, and between STRL33 and chemokine receptors IL-8RB (CXCR2), CXCR4, CCR3, CCR5, and IL-8RA (CXCR1) are 30, 30, 30, 29, and 28%, respectively. *STRL22* is an orphan receptor gene that, like *STRL33*, was isolated from the F9 TIL (22).

Fig. 1 shows that similarities among the receptors are greatest in the TMDs, as is typical of GPCRs. Like other GPCRs, STRL33 includes a site for N-linked glycosylation in the NH_2-terminal domain (N16), cysteines in extracellular loops one and two (C102 and C180), and multiple serines in the COOH terminal domain (1). While there is no signature sequence motif for the chemokine receptors, the STRL33 sequence does contain some features characteristic of chemokine receptors, including an acidic NH_2-terminal domain with paired acidic residues (E8 and D9, E21 and E22) (2), a short basic third intracellular loop, an alanine in place of the proline that is conserved in non-chemokine receptor GPCRs in the second intracellular loop (A134), a paired cysteine and tyrosine in TMD V (C210 and Y211), and a cysteine in TMD VII (C282). In contrast, some residues typical for chemokine receptors are absent from STRL33, including cysteine residues in the NH_2-terminal domain and in the third extracellular loop. Multiple sequence alignment (PileUp, Genetics Computer Group, Madison, WI) places STRL33 in a group of orphan receptors, including STRL22, GPR-9-6, and EBI1, separate from the groupings of the CXCRs on one hand and the CCRs on the other (data not shown).

We analyzed RNA expression by Northern blot of total RNA for *STRL33* and other related receptors in leukocyte

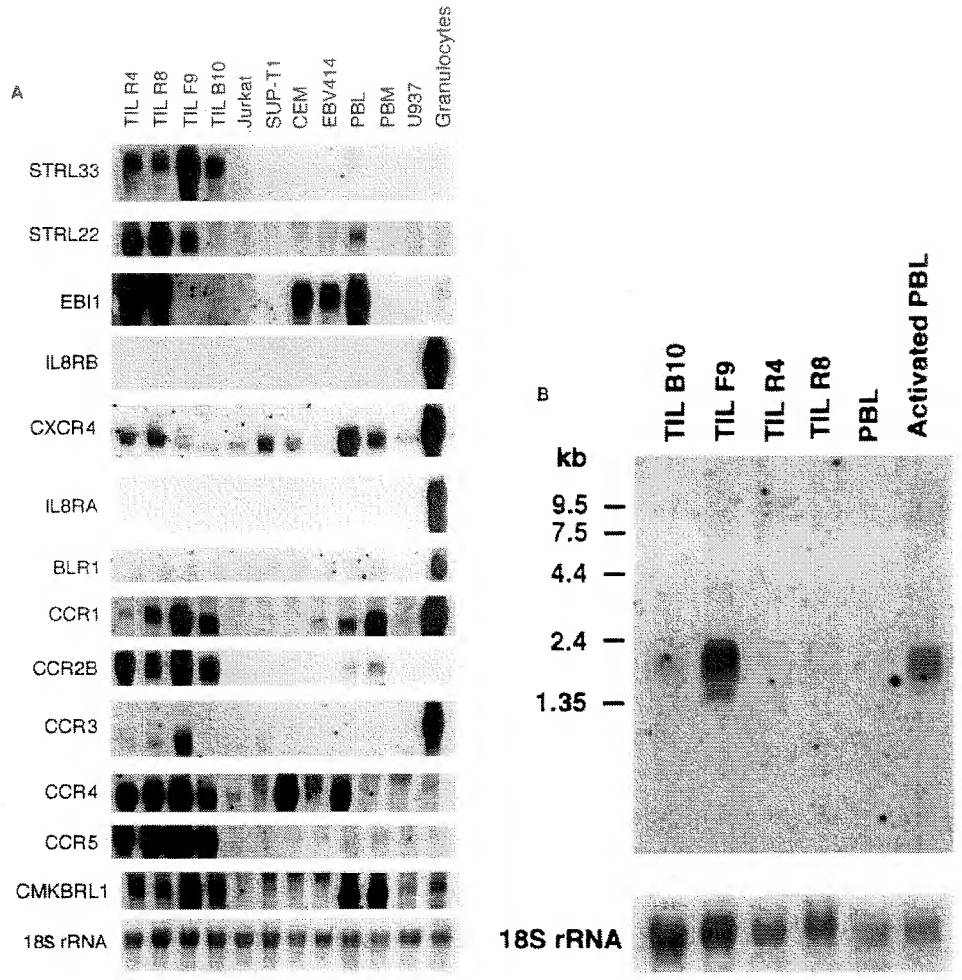


Figure 2. Expression of *STRL33* and other GPCR genes. (*A*) The expression of *STRL33*, genes for known chemokine receptors, and genes for selected orphan GPCRs in leukocytes. 15 μg of total RNA were electrophoresed on 1.2% agarose–formaldehyde gels, transferred to nitrocellulose membranes, and hybridized with the probes indicated on the left. A total of six membranes were used for hybridizations, and adequate removal of signal was documented before repeat probings. Film exposure times ranged from overnight for the IL-8RA and IL-8RB blots to 13 d for the CXCR4 blot. Probings were done using an oligonucleotide complementary to 18S rRNA in order to demonstrate amounts of RNA loaded per lane and a representative blot is shown. (*B*) The expression of *STRL33* in activated PBL. 25 μg of total RNA from TIL, and freshly isolated and activated PBL were analyzed as in *A* and hybridized with a ^{32}P-labeled *STRL33* ORF probe and a probe for 18S rRNA.

populations and lines and in human tissues. As shown in Fig. 2 *A*, the *STRL33* cDNA probe hybridized to a broad band at ~2 kb that was prominent in both CD4$^+$ (R4, F9, and B10) and CD8$^+$ (R8) TIL with low level signal in PBL but not in other cells tested, including immortalized CD4$^+$ T cell lines. Using a probe from the 5′ nontranslated sequences specific for the *STRL33*.3 mRNA, we also detected a band at ~3.6 kb in the F9 and B10 TIL after long exposure, not shown in Fig. 2 *A*. Fig. 2 *B* demonstrates that in vitro activation of PBL induces expression of *STRL33* to levels comparable to that seen in the TIL.

Fig. 2 *A* shows significant differences in expression of various chemokine receptor and orphan receptor genes among the leukocytes. Of particular interest is the demonstration of heterogeneity of receptor gene expression among T cell preparations. In general, receptor gene expression is higher among the TIL than in T cell lines or freshly isolated PBL, although even among the TIL there are significant differences. For example, the CD4$^+$ F9 TIL show a significant signal for CCR3. This is noteworthy, because although CCR3 can serve in vitro as a coreceptor for HIV-1 (13), speculation on a role for CCR3 in systemic HIV-1 infection has been constrained by the assumption that among leukocytes CCR3 expression is limited to eosinophils.

Fig. 3 shows the expression of the *STRL33* gene in selected human tissues. There is an mRNA species of ~2.1 kb prominently expressed in lymphoid tissue; a species of ~2.5 kb in placenta; low abundance species of 2.1-2.4 kb expressed in pancreas, liver, lung, and heart; and low abundance larger species in a variety of tissues. The conspicuous expression of the *STRL33* gene in T cells, activated PBL, and lymphoid tissues is consistent with the presumption that *STRL33* is a chemokine receptor.

HEK 293 cells were transfected with an expression vector containing the *STRL33*.1 ORF, and cell lines were derived as described in Materials and Methods. Cell lines expressing the highest levels of *STRL33* RNA were tested in a fluorometric calcium flux assay for responses to chemokines. The *STRL33*-transfected cells were tested with platelet factor 4, IL-8, IP-10, HuMig, SDF-1, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-2, MCP-3, MCP-4, I309, and lymphotactin, and no responses were found.

The demonstrations that receptors for both CXC and CC chemokines can function as cofactors for HIV-1 entry into cells led us to test STRL33 in an assay designed to de-

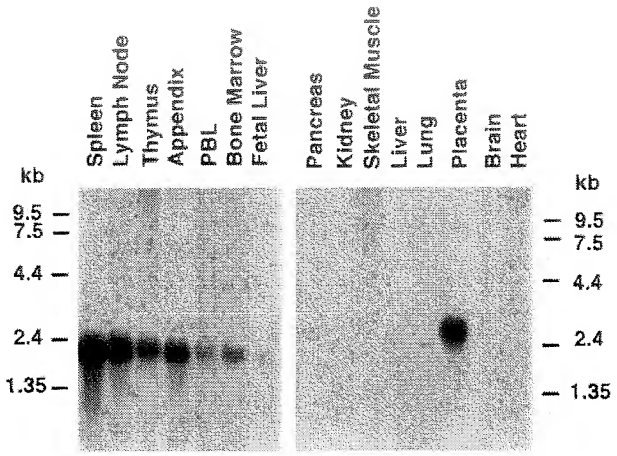
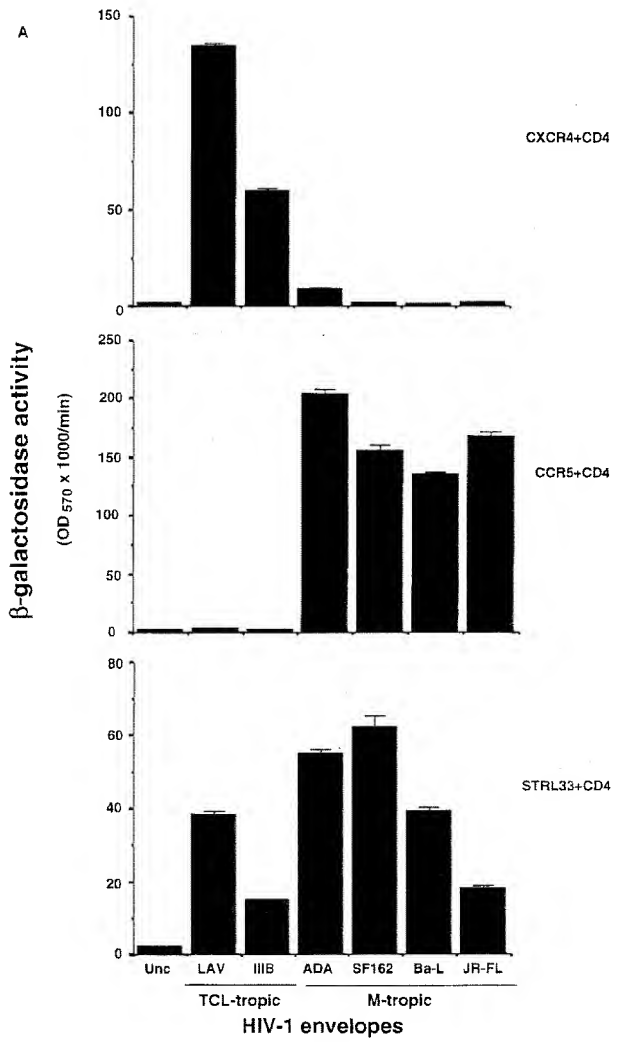


Figure 3. The expression of *STRL33* in human tissues. Blots were prepared by the supplier (Clontech, Palo Alto, CA) from 1.2% agarose–formaldehyde gels containing ~2 μg poly(A)$^+$ RNA per lane. Hybridizations were done using a ^{32}P-labeled *STRL33* ORF probe and blots were washed according to the instructions of the manufacturer. The blot prepared from lymphoid tissue (*left*) was exposed for 2 d, and the blot from other selected tissues (*right*) was exposed for 8 d.

tect fusion between two cell populations: NIH 3T3 cells expressing T7 RNA polymerase, human CD4, and either STRL33 or CXCR4 or CCR5 , and HeLa cells expressing Envs from HIV-1 isolates with differing tropisms. Fusion between the two cell populations resulted in expression of β-Gal. As a negative control, we used the Unc Env, a mutant protein that cannot mediate fusion due to a deletion of the gp120/gp41 cleavage site.

The results of the fusion assays are shown in Fig. 4. In Fig. 4 *A*, NIH 3T3 cells expressing CD4 plus CXCR4 fused well with cells expressing Envs from TCL-tropic LAV (NL4.3) and IIIB, whereas with Envs from the M-tropic strains only weak (ADA) or negligible (SF162, Ba-L, and JR-FL) fusion was observed. Cells expressing CD4 plus CCR5 showed the opposite specificity: they fused well



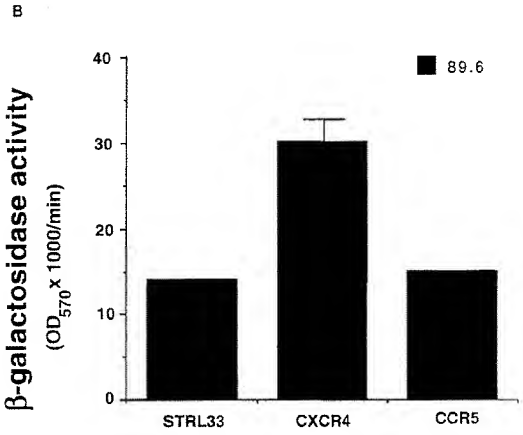


Figure 4. Activity of STRL33 as a fusion cofactor. (*A*) NIH 3T3 cells were transfected with DNAs encoding CXCR4 or CCR5 or STRL33, and infected with vaccinia recombinants encoding CD4 and T7 RNA polymerase. HeLa cells were infected with a vaccinia recombinant containing *lacZ* under control of a T7 promoter and infected separately with vaccinia recombinants encoding the indicated Envs. Unc is a mutant Env that cannot be cleaved to gp120 and gp41 and cannot mediate fusion. Cell fusion was quantified by measuring β-Gal activity. NIH 3T3 cells transfected with the *STRL33* cDNA but not infected with virus vCB-3 encoding CD4 did not fuse with cells expressing any of the Envs (data not shown). Results of one experiment are shown. STRL33 also mediated fusion with cells expressing both TCL-tropic and M-tropic Envs in four additional experiments. (*B*) Cell fusion experiment done as in *A*, except that here the DNA encoding the Env, 89.6, was introduced into HeLa cells by transfection of plasmid DNA, resulting in lower levels of fusion overall as compared with *A*. The value obtained for Unc has been subtracted from each of the values obtained using the 89.6 Env. Results are shown from one experiment. Similar activity for STRL33 was found in another experiment using the 89.6 Env.

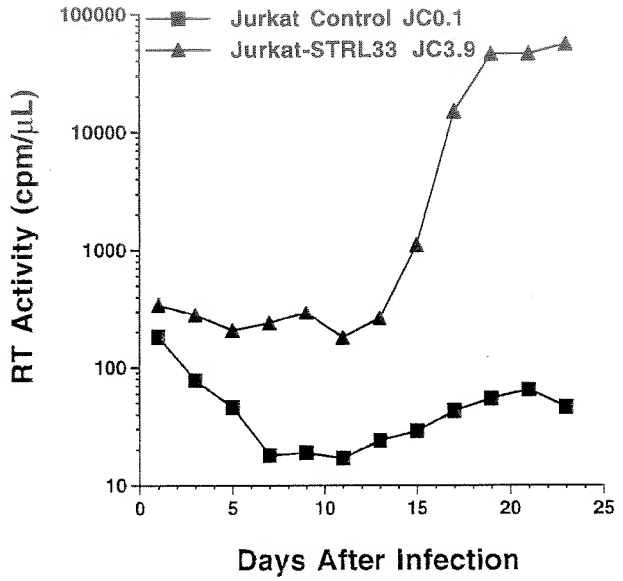


Figure 5. HIV-1 infection of *STRL33*-transfected Jurkat cells. 2×10^6 cells were infected with HIV-1_{ELI} corresponding to 10^5 cpm of RT activity. Samples were taken every 2 d for determining RT activity from cultures of the control-transfected Jurkat cell line JC0.1 (■) and from cultures of the *STRL33*.1-transfected Jurkat cell line JC3.9 (▲).

with cells expressing the Envs from the M-tropic strains ADA, SF162, Ba-L, and JR-FL and not the Envs from TCL-tropic LAV (NL4.3) and IIIB. These results are consistent with previous reports (8, 11–15).

In contrast with the restricted specificities of CXCR4 and CCR5, STRL33 functioned with CD4 as a fusion cofactor for cells expressing Envs from both TCL-tropic and M-tropic strains. Additionally, as shown in Fig. 4 *B*, STRL33, like CXCR4 and CCR5, mediated fusion with the dual-tropic Env 89.6. Negligible β-Gal activity, equivalent to levels seen using the Unc Env, was detected in fusion assays using CD4-expressing NIH 3T3 cells transfected with a control vector lacking the *STRL33* cDNA insert, or in assays using NIH 3T3 cells transfected with the *STRL33* cDNA but not expressing CD4 (data not shown).

We tested the ability of STRL33 to support productive infection with HIV-1. We transfected Jurkat cells, which express CD4 and CXCR4, but not other known HIV-1 coreceptors, with vector containing the *STRL33*.1 cDNA, or with vector control, and cell lines were derived under selection by limiting dilution. We infected the *STRL33*-transfected and control cells with HIV-1_{ELI}, a molecularly cloned isolate with properties of a primary virus (34, 40, 41). HIV-1_{ELI} has been shown to grow poorly in Jurkat cells (34) and CXCR4 does not function efficiently as a coreceptor for the HIV-1_{ELI} Env (13). As shown in Fig. 5, the *STRL33*-transfected cells supported productive infection with HIV-1_{ELI} under conditions where control transfected cells yielded no detectable virus. Although in experiments using higher innocula of virus, HIV-1_{ELI}, as reported (34), could infect cultures of control Jurkat cells, the activity of *STRL33* was also clear in these experiments, because HIV-1_{ELI} production by the control cultures lagged significantly behind HIV-1_{ELI} production in cultures of *STRL33*-transfected cells (data not shown). Enhanced infection with HIV-1_{ELI} in *STRL33*-transfected Jurkat cells was demonstrated in four experiments using a total of three independent *STRL33*-transfected cell lines.

The identification of *STRL33* adds to the recent discoveries on the roles of chemokine receptors in the pathobiology of HIV-1 infection. STRL33 is a novel GPCR that can function with CD4 to mediate fusion with cells bearing HIV-1 Envs from laboratory-adapted TCL-tropic, M-tropic, and dual-tropic strains. In this regard, STRL33 can mediate fusion with a wider range of Envs than can the major cofactors CXCR4 and CCR5. Recent literature has supported the strong correlation between assays for Env/coreceptor–mediated fusion and infection (8, 11–16), and we have demonstrated that STRL33 can significantly enhance the ability of HIV-1 to infect cells. While the role of STRL33 in the biology of viral infection is unknown, the *STRL33* gene is expressed in cells and tissues that are among the natural targets for HIV-1. Further work will determine whether STRL33 represents an important host component in the transmission of HIV-1 and the progression of AIDS.

Thanks are due to J. Yannelli, P. Murphy, S. Ahuja, C. Combadière, Y. Chang, R. Siliciano, and M. Tsang for reagents and/or helpful discussions.

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Received for publication 16 December 1996 and in revised form 9 April 1997.

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A new SIV co-receptor, STRL33

The identification last year of chemokine receptors as fusion co-factors for HIV-1[1–6] has contributed significantly towards understanding HIV transmission and AIDS pathogenesis (see ref. 7 for a review). Because the experimental infection of rhesus macaques with simian immunodeficiency virus (SIV) and the resulting development of an AIDS-like illness is the best animal model for HIV disease in humans[8], identifying SIV co-receptors analogous to those used by HIV has obvious importance. We now report that STRL33, a chemokine receptor-like orphan receptor expressed in activated human lymphocytes and acting as a fusion co-factor with envelope glycoproteins (Envs) from HIV-1 strains of diverse tropisms[9], is a co-receptor for SIV.

The chemokine receptors CXCR4 and CCR5 are known to be important HIV-1 co-receptors for T-cell-line-tropic (TCL-tropic)[1] and macrophage-tropic (M-tropic) isolates[2–6], respectively. The chemokine receptors CCR2B and CCR3 can also act as co-receptors[5,6]. Although CCR5, but not CXCR4, CCR1, CCR2B, CCR3 or CCR4, can serve as a co-receptor for diverse strains of SIV[10–12], it is also clear that human peripheral blood mononuclear cells express an SIV co-receptor(s) distinct from CCR5[10].

First, we assessed SIV co-receptor activity of STRL33 and various chemokine receptors using the vaccinia-based cell fusion assay[13]. We tested cells expressing the Envs from SIV_{mac239} (TCL-tropic) and from SIV_{mac316} (M-tropic) for their ability to fuse with cells expressing CD4 and a single candidate co-receptor. STRL33 has potent fusion co-receptor activity with both SIV Envs (Fig. 1a). Consistent with previous reports[10–12], CCR5 functions with both Envs. We found no SIV_{mac} fusion co-receptor activity with CCR1, CCR2B, CCR3, CXCR4 (Fig. 1a) or CCR4 (not shown).

We next examined the ability of SIV_{mac239} to establish a productive infection in transfectants of human Jurkat T cells stably expressing STRL33 or CCR5. Both STRL33 and CCR5 render Jurkat cells permissive to SIV infection (Fig. 1b), whereas we observed no productive infection in the parental Jurkat cells (Fig. 1b) or in various transfected control cell clones (data not shown). The basis for the difference in replication kinetics in the Jurkat–STRL33 and the Jurkat–CCR5 cultures is unknown — contributing factors could be differences in the levels of co-receptor expression and the efficiencies of co-receptor function for SIV_{mac239}.

The discovery of individuals who are infected with HIV-1 despite being homozygous for an inactivating deletion in the CCR5 gene[14–16] shows that at least one receptor other than CCR5 can be important in HIV disease. Further investigation of STRL33 and other members of the co-receptor repertoire is thus critical for understanding the natural disease process, and may assume added significance if HIV-1 is placed under selective pressure by therapies designed to block a specific co-receptor. Further, the activity of STRL33 with SIV_{mac} has relevance to human AIDS beyond the general parallels between the human and simian systems, as SIV_{mac} is phylogenetically close to, and thought to be the immediate progenitor of, HIV-2 (ref. 17), a virus known to cause AIDS.

Together with our previous report that STRL33 functions with HIV-1 strains of diverse tropisms[9], our present findings with SIV demonstrate that STRL33 is active with a broader range of Envs than has been described for any of the co-receptors so far discovered. This attribute suggests that STRL33 will be of particular value in unravelling the structural determinants of interactions between Envs and co-receptors.

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• STRL33 is identical to Bonzo, one of the SIV co-receptors described by Deng *et al.* elsewhere in this issue[18]. See also N&V, pp. 230–231.

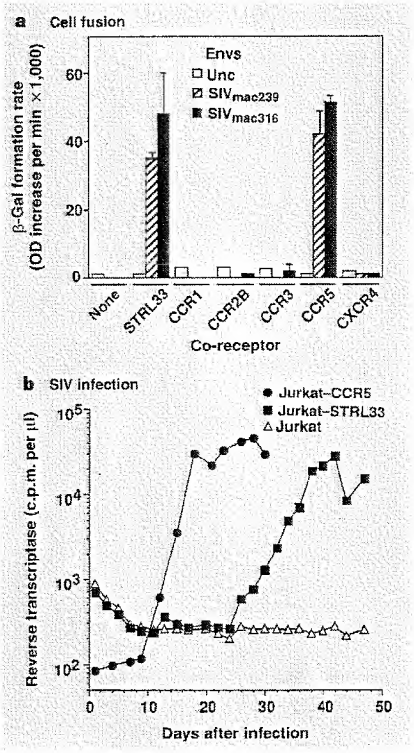

Figure 1 SIV co-receptor activity of STRL33, also referred to as Bonzo[18]. **a,** Vaccinia-based cell fusion assay (see citations in refs1, 4 and 13 for methods). We transfected target cells (NIH 3T3) with plasmids containing the candidate co-receptor complementary DNAs linked to the bacteriophage T7 promoter and then co-infected them with vaccinia recombinant vTF7-3 encoding T7 RNA polymerase and vCB-3 encoding CD4. We co-infected effector cells (HeLa) with vCB-21R (containing the *Escherichia coli LacZ* gene linked to the T7 promoter) plus one of the following Env-encoding vaccinia recombinants: vCB-74 (SIV_{mac239}) or vCB-75 (SIV_{mac316}) (C. C. Broder and E. A. B., personal communication), or vCB-16 (nonfusogenic uncleaved Unc IIIB). We assessed cell fusion after 2 h by colorimetric assay of β-galactosidase activity in detergent cell lysates. **b,** Productive SIV_{mac239} infection of Jurkat cell transfectants. We prepared virus stock from HEK 293 cells transfected with SIV_{mac239} DNA and used it to infect Jurkat cell transfectants stably expressing STRL33 (ref. 9) or CCR5 (ref. 4) (as well as control Jurkat cells). We assessed virus production by measuring reverse transcriptase activity in the medium. The Jurkat–CCR5 infection was done in a separate experiment from other Jurkat infections.

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Two Orphan Seven-Transmembrane Segment Receptors Which Are Expressed in CD4-positive Cells Support Simian Immunodeficiency Virus Infection

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Summary

Clinical isolates of primate immunodeficiency viruses, including human immunodeficiency virus type 1 (HIV-1), enter target cells by sequential binding to CD4 and the chemokine receptor CCR5, a member of the seven-transmembrane receptor family. HIV-1 variants which use additional chemokine receptors are present in the central nervous system or emerge during the course of infection. Simian immunodeficiency viruses (SIV) have been shown to use CCR5 as a coreceptor, but no other receptors for these viruses have been identified. Here we show that two orphan seven-transmembrane segment receptors, gpr1 and gpr15, serve as coreceptors for SIV, and are expressed in human alveolar macrophages. The more efficient of these, gpr15, is also expressed in human $CD4^+$ T lymphocytes and activated rhesus macaque peripheral blood mononuclear cells. The gpr15 and gpr1 proteins lack several hallmarks of chemokine receptors, but share with CCR5 an amino-terminal motif rich in tyrosine residues. These results underscore the potential diversity of seven-transmembrane segment receptors used as entry cofactors by primate immunodeficiency viruses, and may contribute to an understanding of viral variation and pathogenesis.

The human immunodeficiency viruses, HIV-1 and HIV-2, induce acquired immunodeficiency syndrome (AIDS) in humans, and simian immunodeficiency viruses (SIV)[1] can induce AIDS-like illness in Old World monkeys (1–5). Isolates of HIV-1, the major cause of AIDS in humans, have been phylogenetically segregated into groups M and O (6). HIV-2 and SIV form a distinct group of phylogenetically and antigenically related viruses (2, 3, 6–8).

AIDS induced by HIV-1 or HIV-2 in humans or by SIV in monkeys is characterized by the depletion of $CD4^+$ T lymphocytes, which represent a major target of viral infection in vivo (9). Infection of other $CD4^+$ cell types, such as monocytes in the blood, tissue macrophages, and microglial cells in the brain, has been suggested to be important for the pathogenesis of primate immunodeficiency viruses in the central nervous system and in the lungs (10–14). Certain populations of dendritic cells in the blood and tissues may also be infected by these viruses (15, 16).

The tropism of primate immunodeficiency viruses for $CD4^+$ cells is explained by the use of the CD4 glycoprotein as a primary receptor for virus entry into the cell (17–19). The viral envelope glycoproteins, which mediate virus entry, consist of the gp120 exterior envelope glycoprotein and the gp41 transmembrane glycoprotein (20, 21). The gp120 glycoprotein binds the CD4 molecule, following which the gp120–CD4 complex binds one of the members of the chemokine receptor subgroup of seven-transmembrane segment (7-TMS) receptors (22–24). This binding is believed to promote conformational changes in the gp120 and gp41 glycoproteins which result in the fusion of viral and cellular membranes (25–27).

[1] *Abbreviations used in this paper:* 7-TMS, seven-transmembrane segment; CAT, chloramphenicol acetyltransferase; SIV, simian immunodeficiency virus.

M. Farzan and H. Choe contributed equally to this work.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/08/405/07 $2.00
Volume 186, Number 3, August 4, 1997 405–411
http://www.jem.org

Viral variation, particularly that found in the gp120 glycoprotein sequences (28, 29), dictates the specific chemokine receptor which can be used as an entry cofactor. M-tropic HIV-1 variants which use the chemokine receptor CCR5 as a coreceptor predominate during the asymptomatic stages of infection (30–35). CCR5 is expressed on T lymphocytes, monocytes/macrophages, brain microglia, and dendritic cells (36–39). Individuals with defects in CCR5 expression are relatively resistant to HIV-1 infection (40–42), indicating the critical contribution of this chemokine receptor to virus transmission. Some M-tropic brain isolates of HIV-1 also use the chemokine receptor CCR3 as a coreceptor, consistent with the expression of CCR3 in brain microglia (39). Later in the course of infection, T-tropic HIV-1 variants emerge which can use chemokine receptors, especially CXCR4, but also CCR3 and CCR2b, in addition to CCR5 (34, 35, 43–45). The emergence of these viruses has been suggested to coincide with a less favorable clinical prognosis (45), perhaps through an expansion of the range of infectable $CD4^+$ T cell subsets (46).

Primary isolates of HIV-2 and SIV have been shown to use rhesus macaque or human CCR5 as a coreceptor (47, 48) and are inhibited by the natural CCR5 ligands, MIP-1α, MIP-1β, and RANTES (49). None of the other known HIV-1 coreceptors has been shown to be used by SIV, whereas some isolates of HIV-2 can use CXCR4 for entry into $CD4^-$ cells (50). Several lines of evidence have suggested the existence of at least one other coreceptor for SIV. A human B cell/T cell hybrid, CEM×174, supports SIV entry, but lacks CCR5 and does not support efficient entry of HIV-1 viruses using CCR5 (48). A neuroglioma cell line, U87, stably transfected with CD4, similarly supports entry of $SIV_{mac}239$ but does not allow for efficient entry of any known HIV-1 virus (51). Finally, PBMCs from humans lacking a functional CCR5 receptor can nonetheless be infected with SIV (48). Here we identify two additional SIV coreceptors, gpr1 and gpr15, which are expressed in U87 and CEM×174 cells, respectively. Both proteins are expressed in human alveolar macrophages, and the gpr15 protein is also expressed in $CD4^+$ T lymphocytes.

Materials and Methods

Preparation of cDNA Libraries and cDNA. Messenger RNA was isolated using the CsCl method and selection on magnetic beads with oligo-dT (Dynabeads; DYNAL, Inc., Lake Success, NY). RNA was obtained from purified human $CD4^+$ peripheral blood T cells (gift of Dr. Linda Clayton, Dana-Farber Cancer Institutes, Boston, MA), human alveolar macrophages (gift of Dr. Hal Chapman, Brigham and Women's Hospital, Boston, MA), CEM×174 cells, and U87 neuroglioma cells. RNA was also isolated from phytohemagglutinin-treated, interleukin-2–stimulated PBMCs from a healthy rhesus macaque (New England Regional Primate Research Center, Foxboro, MA). The cDNA libraries from the U87 and CEM×174 cell lines were made by reverse transcription (Superscript; GIBCO BRL, Gaithersburg, MD) using a unidirectional primer supplied by the manufacturer. Size-selected cDNAs were cloned into a BstXI/NotI-digested pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The human alveolar macrophage library was prepared by Invitrogen in pcDNA1. Double-stranded $CD4^+$ T cell cDNA was synthesized using a kit from Boehringer Mannheim (Indianapolis, IN).

Cloning of cDNAs for 7-TMS Proteins. The expression plasmids for rdc1, ebi2, gpr1, gpr15, and dez were prepared by PCR amplification of a cDNA library made from either CEM×174 or U87 cells, as described above. The amplified fragments were cloned into the pcDNA3 plasmid for expression. Expression plasmids for other chemokine receptors were generously supplied by Drs. Paul Ponath and Walter Newman (LeukoSite, Inc., Cambridge, MA) (v28, CXCR1, CXCR2), Dr. Elliot Kieff (Harvard Medical School, Boston, MA) (ebi1), and Dr. Monica Napolitano (Regina Elena Cancer Institut, Rome, Italy) (ter1).

Testing SIV Coreceptor Activity. A previously described *env*-complementation method (27, 28, 47) was used to produce recombinant HIV-1 viruses which contained the SIV envelope glycoproteins and were capable of encoding chloramphenicol acetyltransferase (CAT) in target cells. Briefly, recombinant virus was incubated with Cf2Th cells transfected with plasmids expressing human CD4 and candidate coreceptors. Cells were har-

Table 1. *Expression of 7-TMS Proteins and Activity as an $SIV_{mac}239$ Coreceptor*

7-TMS protein	Reference	Expression in: CEM×174	U87	Primary $CD4^+$ T cells	$SIV_{mac}239$ coreceptor activity
apj	55	−	−	−	ND
blr1	56	+	−	ND	−
CCR5	38, 57	−	−	+	+
CXCR1	58	ND	ND	ND	−
CXCR2	59	ND	ND	ND	−
CXCR3	60	ND	ND	ND	−
CXCR4	61	+	+	+	−
dez	62	−	+	+	−
ebi1	63	+	+	+	−
ebi2	63	+	+	+	−
gc96	*	−	−	+	ND
gcy4	64	−	−	+	ND
gpr1	53	−	+	−	+
gpr2	53	−	−	−	ND
gpr4	65	−	−	+	ND
gpr5	65	−	−	−	ND
gpr15	54	+	−	+	+
rdc1	66	+	+	+	−
ter1	67	+	−	+	−
v28	68	ND	ND	ND	−

Positive expression values indicate the detection of a PCR product of expected size and restriction map amplified from the indicated cDNA source. Coreceptor activity for viruses with $SIV_{mac}239$ envelope glycoproteins was determined as described in Materials and Methods.
*These sequence data are available from EMBL/GenBank/DDBJ under accession number U45982.

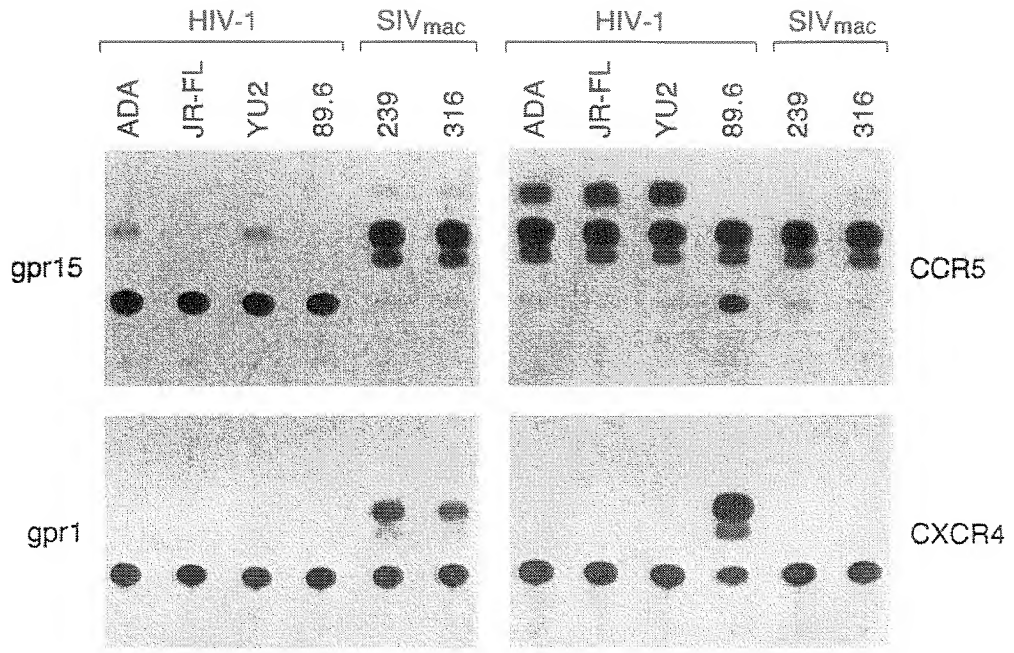

Figure 1. CAT activity in Cf2Th cells expressing CD4 alone or together with gpr1, gpr15, CCR5, or CXCR4 after incubation with HIV-1 recombinant viruses carrying the $SIV_{mac}239$, $SIV_{mac}316$, or HIV-1 (YU2, HXBc2, 89.6, or ADA) envelope glycoproteins. A representative experiment is shown. The amount of target cell lysate used was equivalent for all the experiments shown. CAT activity was determined by calculating the percentage of chloramphenicol present in acetylated forms (*three uppermost spots*) to the total amount of chloramphenicol. The nonacetylated form of chloramphenicol is present in the spot closest to the origin, which is near the bottom of the figure.

vested and assayed for CAT activity, which was determined by measuring the conversion of chloramphenicol to acetylated forms of chloramphenicol. The $SIV_{mac}239$ and $SIV_{mac}316$ envelope glycoproteins were expressed from the previously described pSIVΔgpv plasmid (47). The HIV-1 envelope glycoproteins were expressed as previously described (27, 28).

Analysis of Expression of 7-TMS Proteins in Cells and Tissues. The expression of 7-TMS protein messenger RNA in CEM×174 and U87 cells and primary human $CD4^+$ T lymphocytes and alveolar macrophages was examined by synthesis of cDNA from polyadenylated RNA prepared from these cells, as described above. Primers corresponding to the nucleotide sequences encoding the first and third extracellular loops of the proteins were used for amplification by PCR. The identity of amplified fragments was confirmed by restriction enzyme digestion.

Results

We had previously tested a number of human chemokine receptors (CCR1–CCR5, as well as CXCR4) and found that of these only CCR5 could support entry of an HIV-1 virus pseudotyped with the envelope glycoproteins of a pathogenic, molecularly cloned SIV, $SIV_{mac}239$ (47). To identify additional coreceptors which might be used by SIV, we screened cDNA libraries from SIV-infectable cells, CEM×174 and U87, for the expression of mRNA encoding known 7-TMS proteins exhibiting some sequence similarity to chemokine receptors. The cDNAs which were shown to be expressed in either cell line were tested for the ability to support SIV and HIV-1 entry. Recombinant HIV-1 viruses which contained either HIV-1 or SIV envelope glycoproteins and expressed CAT were incubated with Cf2Th canine thymocytes transfected with plasmids expressing human CD4 and the 7-TMS proteins. Table 1 lists the 7-TMS proteins tested, summarizes their expression in CEM×174, U87, and human $CD4^+$ T cells, and indicates coreceptor activity for viruses with the $SIV_{mac}239$ envelope glycoproteins. Of the 7-TMS proteins tested, only gpr1, gpr15, and CCR5 supported the entry of viruses with the

Table 2. *CAT Activity in Cf2Th Cells Expressing CD4 and 7-TMS Proteins following Incubation with Viruses Containing Different Envelope Glycoproteins*

	Viral envelope glycoproteins					
	SIV		HIV-1			
7-TMS protein	$SIV_{mac}239$	$SIV_{mac}316$	ADA	YU2	JR-FL	89.6
CXCR4	<0.1	<0.1	<0.1	<0.1	<0.1	9.3
CCR5	19.0	12.1	113.9	290.8	203.7	9.4
gpr1	7.0	2.7	<0.1	<0.1	<0.1	<0.1
gpr15	30.3	30.5	0.7	0.9	<0.1	<0.1

The percent conversion of chloramphenicol to acetylated forms is shown following incubation of comparable amounts of lysates derived from Cf2Th cells exposed to recombinant viruses. The CAT activity was calculated as described in the legend to Fig. 1. In some cases, dilutions of the lysates were tested to bring the assay within the linear range and, thus, the reported values exceed 100%.

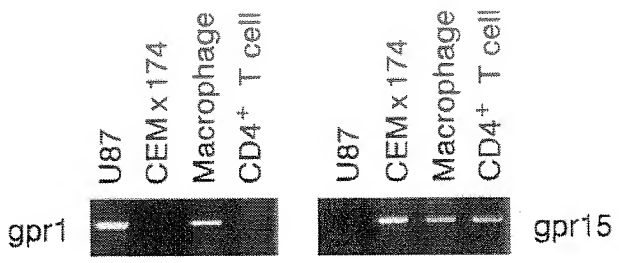

Figure 2. Expression of gpr1 and gpr15 RNA in cells. The cDNA libraries from U87 and CEM×174 cells and from human alveolar macrophages, as well as cDNA prepared from human $CD4^+$ T lymphocytes, were PCR amplified using gpr1- or gpr15-specific primers.

$SIV_{mac}239$ envelope glycoproteins. These three 7-TMS proteins also supported the entry of viruses with the macrophage-tropic $SIV_{mac}316$ envelope glycoproteins (Fig. 1). The SIV coreceptor activity exhibited by the gpr15 protein was greater than that of CCR5, whereas the coreceptor activity of gpr1 was ~30% that of CCR5 (Table 2). Most of the viruses with HIV-1 envelope glycoproteins (HXBc2, JR-FL, 89.6) did not infect Cf2Th cells expressing CD4 and gpr15, although the viruses with the M-tropic HIV-1 ADA and YU2 envelope glycoproteins demonstrated a low but reproducible signal in these cells (Fig. 1 and Table 2). Following incubation with the ADA and YU2 viruses, the CAT conversion in the $CD4^+$, $gpr15^+$ Cf2Th cells was <1% of that seen in the $CD4^+$, $CCR5^+$ control cells (Table 2). Cf2Th cells expressing CD4 and gpr1 were not infected by viruses containing any of the HIV-1 envelope glycoproteins tested (Table 2).

The expression of gpr15 and gpr1 in different cell types was examined. Since specific reagents to detect these proteins were not available, expression was examined by RNA analysis. A cDNA for gpr15 was readily detected in human $CD4^+$ T lymphocytes, in human alveolar macrophages, in activated rhesus macaque PBMCs, and in CEM×174 cells, but not in U87 cells (Table 1, Fig. 2, and data not shown). By contrast, a cDNA for gpr1 could not be detected in primary human T lymphocytes, activated rhesus macaque PBMCs, or CEM×174 cells, but was detected in U87 cells and human alveolar macrophages.

Discussion

HIV-1, HIV-2, and SIV all use CCR5 as a coreceptor, indicating the importance of this protein in primate immunodeficiency virus pathogenesis (28, 30–33, 47, 48). The use of other chemokine receptors by HIV-1 has been suggested to be important for infection of anatomical compartments such as the brain or for more efficient T cell depletion (39, 45, 46). The identification of gpr1 and gpr15 as additional SIV coreceptors should assist efforts to understand the consequences of the use of coreceptors other than CCR5 in primate models of AIDS. While the in vivo contribution of gpr15 to SIV replication and pathogenesis requires further investigation, several lines of evidence indicate that gpr15 is an important SIV coreceptor. The gpr15 protein is expressed on $CD4^+$ T lymphocytes, a major target cell for SIV infection in vivo (10), and on alveolar macrophages. The gpr15 protein is also expressed on CEM× 174 cells, which are routinely used to passage SIV obtained from monkey PBMCs (52). The rapid outgrowth of SIV viruses with minimal sequence changes on CEM×174 cells suggests that these cells express a receptor used by primary SIV viruses. The weak use of gpr15 by the ADA and YU2 HIV-1 viruses may be an inadvertent consequence of similarities in the amino-terminal regions of gpr15 and CCR5, or may indicate that adaptation to these receptors or to a related receptor occurs in some subsets of HIV-1.

```
gpr15          MDPEETSVYLDYYYATSPN
gpr1     MEDLEETLFEEFENYSYDLDYYSLESD
rCCR5          MDYQVSSPTYDIDYYTSEPC
CCR5           MDYQVSSPIYDINYYTSEPC
```

Figure 3. An alignment of human gpr1, human gpr15, rhesus CCR5 (rccr5), and human CCR5 from the NH_2-terminus through the first cysteine of CCR5 is shown. Tyrosines shown to be important for HIV-1 and $SIV_{mac}239$ entry are shown in bold. Other residues similarly positioned in these proteins are underlined. Sequences for gpr1 and gpr15 are provided in references 53 and 54, respectively.

The in vivo contribution of gpr1 to primate immunodeficiency virus infection is also unresolved. The gpr1 protein weakly supported SIV infection in our studies. Whether this inefficient coreceptor activity is an intrinsic property of gpr1 or merely reflects low cell surface expression of gpr1 requires further investigation. While gpr1 is not apparently expressed on primary $CD4^+$ lymphocytes, it is expressed on tissue macrophages and in the brain (53) and thus may play a role in SIV infection of particular nonlymphoid target cells.

In primary structure, gpr1 and gpr15 resemble the angiotensin II receptor and the orphan receptors dez and apj more than they do any of the known chemokine receptors (53, 54). Gpr15, like dez and gpr1, lacks the cysteines in the NH_2-terminal region and the third extracellular loop which, in the chemokine receptors, are thought to be disulfide linked. It is interesting that despite the general sequence divergence of gpr15/gpr1 and other identified primate immunodeficiency virus coreceptors the gpr15 and gpr1 amino termini contain three tyrosines which align with similarly positioned tyrosines in CCR5 (Fig. 3). Alteration of these tyrosines has been shown to decrease the efficiency with which CCR5 supports the entry of SIV and M-tropic HIV-1 isolates (Farzan, M., H. Choe, and J. Sodroski, unpublished observations). The identification of gpr15 and gpr1 as SIV coreceptors suggests a greater range and complexity of coreceptors for the primate immunodeficiency viruses than previously described. Comparative studies of these divergent coreceptors with the known coreceptors for these viruses should assist in the identification of common structural elements in 7-TMS proteins which serve as viral entry cofactors.

We thank Dr. Ronald Desrosiers for the gift of the $SIV_{mac}239$ and $SIV_{mac}316$ infectious proviral clones and Drs. Paul Ponath, Walter Newman, Elliot Kieff, and Monica Napolitano for plasmids expressing chemokine receptors. We thank Ms. Lorraine Rabb and Ms. Yvette McLaughlin for manuscript preparation.

This work was supported by a grant to Joseph Sodroski from the National Institutes of Health (AI-24755) and by a Center for AIDS Research grant to the Dana-Farber Cancer Institute (AI-28691). Dana-Farber Cancer Institute is also the recipient of a Cancer Center grant from the National Institutes of Health (CA-06516). Luisa Marcon was supported by National Cancer Institute National Research Science Award Training Grant (CA-09382) and by an award from Istituto Superiore di Sanitá and by the University of Padua. Craig Gerard was supported by National Institutes of Health grants HL-51366 and AI-36162 as well as by the Rubenstein/Cable Fund at the Perlmutter Laboratory. This work was made possible by gifts from the late William McCarty-Cooper, from the G. Harold and Leila Y. Mathers Charitable Foundation, and from the Friends 10.

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Received for publication 7 May 1997 and in revised form 9 June 1997.

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